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Substitute Specification

Fusion polypeptides, and use thereof in antivascular tumor therapy

The present invention relates to fusion polypeptides, comprising at least two peptides. One peptide comprises from 3 to 30 amino acids and permits the fusion polypeptide to be bound selectively to endothelial cells in tumor vessels. The other peptide consists of the tissue factor (TF) or a fragment thereof, the tissue factor and the fragment being characterized in that they are able to activate blood clotting upon binding of the fusion polypeptide to endothelial cells in tumor vessels. The peptides can be joined together either directly or via a linker having up to 15 amino acids. The invention further relates to the use of these fusion proteins in antivascular therapy of neoplastic diseases and to their use in the production of a drug for the treatment of neoplastic diseases.

Background of the invention

Adequate neovascularization is a prerequisite for progressive tumor growth (1). Neoangiogenesis is required in particular for maintaining expansive tumor growth, since only sufficient oxygenation will ensure the supply with nutrients to and removal of tumor degradation products from the tumor.

In the prior art directed to tumor treatment antivascular therapeutic strategies have been developed, which are aimed at destruction of the tumor blood vessels and associated tumor infarction, in addition to anti-angiogenic therapeutic strategies, which attack the complex process of growth and differentiation of blood vessels.

A precondition for these strategies is identification of target structures in the vascular endothelium of the tumor that do not occur on resting endothelial cells in normal tissue. These specific target structures could be utilized in order to apply cytostatics or certain toxins to the vascular endothelial cells of the tumor to a lesser extent to the tumor cells themselves. Target structures that can be used for this purpose are bFGF (basic fibroblast growth factor), VEGF (vascular endothelial growth factor) and VEGFR-2 (VEGF receptor 2), endoglin, endosialin, a fibronectin isoform (ED-B domains), the integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_1\beta_1$ and $\alpha_1\beta_2$, aminopeptidase N, NG2 proteoglycan and the matrix metalloproteinases 2 and 9 (MMP 2 and 9) (2-13). For example, Arap *et al.* (8) coupled peptides that bind alpha1-integrins specifically, to an active substance that was being used in the state of the art for chemotherapy (doxorubicin). It was demonstrated in an animal model that the antineoplastic effect of doxorubicin could be

improved by coupling to the peptides.

An alternative antivascular therapeutic approach comprises selective activation of blood clotting in tumor vessels, in order to induce tumor necrosis. For example, a bispecific F(ab')2 antibody fragment was produced, which is directed against truncated tissue factor (tTF) and an MHC class II antigen. After experimental induction of the antigen in tumor endothelial cells, an antivascular therapy could be demonstrated by administering the antibody in a murine neuroblastoma model (14). In a second study by the same team, an immunoconjugate was used, which couples tTF selectively to a naturally occurring marker of the tumor vessel endothelium, VCAM-1 (vascular cell adhesion molecule-1) (15).

In a very similar approach, an antibody fragment (scFv), which is specific for the oncofetal ED-B domain, was fused with tTF. The fusion proteins generated, scFv-tTF, led to a complete and selective infarction in various tumors in the mouse model (16).

Alternatively, tTF was coupled to an inhibitor of the prostate-specific membrane antigen (17). This fusion protein induced selective infarction necrosis in a rat prostate model after intravenous administration. Administering this fusion protein in combination with a cytotoxic substance (doxorubicin) at low dose resulted in massive tumor regression and even complete tumor eradication (17). Other tTF fusion proteins, consisting of antibody fragments against VEGFR (VEGF receptor), endoglin and VCAM-1, have been described recently (18).

However, the molecules produced for antivascular tumor therapy in the state of the art have drawbacks. In particular it has to be assumed that these molecules are immunogenic owing to their size. Treatment of mammals with these molecules will therefore trigger an immune reaction against the molecules, so that repeated administration of the molecules becomes impossible.

The size of the coupling partner, by means of which the peptide portion, which can activate blood clotting, is to be directed onto the tumor tissue, may further cause steric hindrance to a formation of the macromolecular factor VIIa/FX enzyme-substrate complex, which is important for blood clotting. Formation of the complex can also be hampered when the peptide capable of activating blood clotting has an altered conformation owing to the relatively large fusion partners.

In the state of the art (WO 03/035688), fusion polypeptides are also known wherein a selective binding domain, e.g. a domain of fibronectin that binds to integrins, e.g. which comprises RGD peptides, or the D-β-E dipeptide, which binds to PSMA (prostate-specific membrane antigen), is coupled to the N-terminus of a tissue factor polypeptide. Although an amidolytic and proteolytic effect was demonstrated *in vitro*, the constructs, even in combination with factor VIIa, only displayed extremely weak anti-tumor effect *in vivo*. The animals only survived longer in combination with doxycycline.

Hu *et al.* (46) describe various fusion proteins and use thereof for the production of thromboses in tumor vessels, including a fusion protein from an oligopeptide with 9 amino acids, containing the RGD sequence, which was coupled to the truncated form of the tissue factor. Again, the RGD peptides were linked to the N-terminus of tTF to obtain RGD-tTF. Functional analysis showed that the fusion protein containing RGD did not produce any significant inhibition of tumor growth.

The constructs known in the state of the art were thus constructed in such a manner that the selective binding domain was linked to the N-terminus of the tissue factor polypeptide. It was even emphasized that this structure must be chosen because the N-terminus, on the basis of structural models, was considered to be an especially favorable site for linkage, which would not inhibit the initiation of thrombosis.

Summary of the invention

In view of this prior art, the problem therefore resides in providing alternative thrombogenic substances, which can effectively inhibit tumor growth in vivo.

This problem is now solved by fusion polypeptides, which comprise a peptide of 3-30 amino acids, which permits the fusion polypeptide to be bound selectively to tumor vessel endothelial cells, and the tissue factor (TF) or a fragment thereof, the tissue factor and the fragment being characterized in that they are able to activate blood clotting when the fusion polypeptide binds to tumor vessel endothelial cells, these peptides being coupled to one another either directly or via a linker having up to 15 amino acids. The peptide, which enables the fusion polypeptide to be bound selectively to tumor vessel endothelial cells, is coupled to the C-terminus of the peptide, which can activate blood clotting when the fusion polypeptide binds to tumor vessel endothelial cells. The present invention further relates to pharmaceutical

compositions containing corresponding fusion polypeptides, and use thereof for the treatment of tumors.

Description of the Figures

Fig. 1: Schematic presentation of binding of the tTF-RGD and tTF-NGR fusion proteins to $\alpha_v\beta_3$ and CD13. Tumor selectivity is achieved owing to the specificity of the RGD sequence for $\alpha_v\beta_3$-integrin and of the NGR sequence for CD13 (aminopeptidase N). These receptors are expressed selectively and specifically at high density on tumor endothelial cells, but not on resting endothelial cells in normal tissue (apart from a few exceptions). The representation of the fusion proteins is highly schematic and does not provide any information regarding the primary sequence.

Fig. 2: SDS-PAGE and Western Blot analysis of recombinant $tTF_{1\text{-}218}$ (SEQ ID NO:2) and tTF fusion proteins. The purity of the tTF and of the tTF fusion proteins was checked by SDS-PAGE and staining with Coomassie Blue after extraction from *E. coli* (BL21 DE3) and refolding over a linear urea gradient (6M – 1M). Identity of the proteins was verified by Western blotting using a monoclonal anti-tissue-factor antibody (clone V1C7, American Diagnostics). Loading in the individual lanes: 1=tTF; 2=tTF-RGD; 3=tTF-NGR; 4=tTF-cycloNGR1 (SEQ ID NO:6); 5=tTF-cycloNGR2 (SEQ ID NO:7); 6=tTF-cycloNGR3 (SEQ ID NO:8); 7=tTF-GALNGRSHAG (SEQ ID NO:5); M = molecular weight marker.

Fig. 3: Determination of the Michaelis constants (Km) for the activation of FX by FVIIa/$tTF_{1\text{-}218}$ or FVIIa/$tTF_{1\text{-}218}$ fusion proteins. The parameters of Michaelis-Menten kinetics were calculated using the method described by Ruf (45).

Fig. 4: Binding of tTF, tTF-RGD and tTF-NGR to integrin $\alpha_v\beta_3$. The binding of 0.1 μM tTF, tTF-RGD and tTF-NGR to immobilized $\alpha_v\beta_3$ was quantified with a polyclonal antibody against human TF (American Diagnostica) in an ELISA. The results are presented as median and interquartile range. The differences in binding between tTF-RGD and tTF or between tTF-NGR and tTF were statistically significant ($p<0.001$, Mann-Whitney test).

Fig. 5: Specificity of the binding of tTF-RGD to integrin $\alpha_v\beta_3$. The binding of tTF-RGD (0.1 μM) to immobilized $\alpha_v\beta_3$ was inhibited significantly by competitive inhibition with the synthetic peptide GRGDSP (SEQ ID NO:33) (1-10 μM) ($p<0.001$, Mann-Whitney test for both

RGD peptide concentrations).

Fig. 6: Binding of tTF and tTF-RGD to human endothelial cells. A: FACS analysis of endothelial cells incubated with 0.1 µM tTF (2) or with 0.1 µM tTF-RGD (3) for 60 min at 4°C. B: A 75% reduction in binding was demonstrated by competitive inhibition of the tTF-RGD fusion protein with 1 µM GRGDSP (SEQ ID NO:33) (4). Curves 1 in A and B show the negative control.

Fig. 7: Inhibition of a human lung carcinoma (CCL185) growing as a xenograft in athymic nude mice by intravenous therapy with tTF fusion proteins (tTF-RGD, n=6; tTF-NGR, n=6) compared with tumor growth with infusion of physiological saline solution (NaCl, n=8) or tTF (n=1). The vertical arrows indicate the times of injection with the respective substances.

Fig. 8: Inhibition and partial remission of a human malignant melanoma (M21) growing as a xenograft in athymic nude mice by intravenous therapy with tTF fusion proteins (tTF-RGD, n=3; tTF-NGR, n=3) compared with tumor growth with infusion of physiological saline solution (NaCl, n=4) or tTF (n=4). The vertical arrows indicate the times of injection with the respective substances.

Fig. 9: Macroscopic *in vivo* photograph of a tumor-bearing mouse 20 min after injection of the tTF-NGR fusion protein (A, left half of the picture) or NaCl (A, right half of the picture). The macroscopic picture with bluish-livid coloration of the tumor after injection of tTF-NGR is indicative of tumor necrosis. After 60 min, the two mice were exsanguinated, the tumor was excised *in toto* and examined histologically. In B, we can see the hemorrhagic imbibition of the tumor treated with tTF-NGR as a sign of secondary hemorrhage as a result of incipient tumor necrosis. In contrast, the NaCl-treated tumor appears to be vital (C).

Fig. 10: Histology of the melanoma tumor 1 hour after intravenous injection of tTF-RGD (A and B), tTF-NGR (C and D) and common salt (E and F) in the caudal vein of the tumor-bearing nude mouse. In the tumors treated with the tTF fusion proteins, the blood vessels appear to be thrombolytically occluded (arrows). Extensive tumor necroses are observable in the supply region of the vessel occluded by a blood clot (A-D). The photographs are of representative areas of the tumors (A, C and E: 200x magnification, B, D and F 400x magnification; HE staining (staining described e.g. in H.C. Burck, Histologische Technik – Leitfaden für die Herstellung mikroskopischer Präparate in Unterricht und Praxis, 5th edition, Thieme Verlag, Stuttgart 1982,

pages 109 ff.).

Fig. 11: Representative histologies of heart (A), kidney (B), liver (C) and lung (D) 1 hour after injection of 4 mg/kg BW tTF-NGR. Thromboses or necroses were not detected microscopically in any of these organs. (HE staining; 200x magnification).

Fig. 12: Amino acid sequence of human tissue factor (TF) (SEQ ID NO:1).

Fig. 13: Amino acid sequence of the truncated human tissue factor $tTF_{1\text{-}218}$ (SEQ ID NO:2) (also designated tTF for short within the scope of the present application).

Fig. 14: Amino acid sequence of the fusion polypeptide tTF-GRGDSP (SEQ ID NO:3) (also abbreviated to tTF-RGD).

Fig. 15: Amino acid sequence of the fusion polypeptide tTF-GNGRAHA (SEQ ID NO:4) (also abbreviated to tTF-NGR).

Fig. 16: Amino acid sequence of the fusion polypeptide tTF-GALNGRSHAG (SEQ ID NO:5).

Fig. 17: Amino acid sequence of the fusion polypeptide tTF-GCNGRCG (SEQ ID NO:6) (also abbreviated to tTF-cycloNGR1).

Fig. 18: Amino acid sequence of the fusion polypeptide tTF-GCNGRCVSGCAGRC (SEQ ID NO:7) (also abbreviated to tTF-cycloNGR2).

Fig. 19: Amino acid sequence of the fusion polypeptide tTF-GCVLNGRMEC (SEQ ID NO:8) (also abbreviated to tTF-cycloNGR3).

Fig. 20: Nucleotide sequence of the truncated human tissue factor $tTF_{1\text{-}218}$ (SEQ ID NO:9) (also designated tTF for short within the scope of the present application).

Fig. 21: Nucleotide sequence of the fusion polypeptide tTF-GRGDSP (SEQ ID NO:10) (also abbreviated to tTF-RGD).

Fig. 22: Nucleotide sequence of the fusion polypeptide tTF-GNGRAHA (SEQ ID NO:11) (also abbreviated to tTF-NGR).

Fig. 23: Nucleotide sequence of the fusion polypeptide tTF-GALNGRSHAG (SEQ ID NO:12).

Fig. 24: Nucleotide sequence of the fusion polypeptide tTF-GCNGRCG (SEQ ID NO:13) (also abbreviated to tTF-cycloNGR1).

Fig. 25: Nucleotide sequence of the fusion polypeptide tTF-GCNGRCVSGCAGRC (SEQ ID NO:14) (also abbreviated to tTF-cycloNGR2).

Fig. 26: Nucleotide sequence of the fusion polypeptide tTF-GCVLNGRMEC (SEQ ID NO:15) (also abbreviated to tTF-cycloNGR3).

Fig. 27: Nucleotide sequence of the oligonucleotides for production of tTF_{1-218} (SEQ ID NO:2).

A: 5'-primer (SEQ ID NO:16); B: 3'-primer (SEQ ID NO:17).

Fig. 28: Nucleotide sequence of the oligonucleotides for production of tTF-GRGDSP (SEQ ID NO:3).

A: 5'-primer (SEQ ID NO:18); B: 3'-primer (SEQ ID NO:19).

Fig. 29: Nucleotide sequence of the oligonucleotides for production of tTF-GNGRAHA (SEQ ID NO:4).

A: 5'-primer (SEQ ID NO:20); B: 3'-primer (SEQ ID NO:21).

Fig. 30: Nucleotide sequence of the oligonucleotides for production of tTF-GCNGRCG (SEQ ID NO:6).

A: 5'-primer (SEQ ID NO:22); B: 3'-primer (SEQ ID NO:23).

Fig. 31: Nucleotide sequence of the oligonucleotides for production of tTF-GCNGRCVSGCAGRC (SEQ ID NO:7). A: 5'-primer (SEQ ID NO:24); B: 3'-primer (SEQ ID NO:25).

Fig. 32: Nucleotide sequence of the oligonucleotides for production of tTF-GCVLNGRMEC (SEQ ID NO:8).

A: 5'-primer (SEQ ID NO:26); B: 3'-primer (SEQ ID NO:27).

Fig. 33: Nucleotide sequence of the oligonucleotides for production of tTF-GALNGRSHAG (SEQ ID NO:5).

A: 5'-primer (SEQ ID NO:28); B: 3'-primer (SEQ ID NO:29).

Fig. 34: a: Inhibition and partial remission of a human malignant melanoma (M21) growing as a xenograft in athymic nude mice by intravenous therapy with tTF fusion proteins (tTF-RGD, n=7) compared with the growth of the tumors with infusion of physiological saline solution (NaCl, n=9) or tTF (n=11). The vertical arrows indicate the times of the injections with the respective substances.

b: Inhibition of a human fibrosarcoma (HT1080) growing as a xenograft in athymic nude mice by intravenous therapy with tTF fusion proteins (tTF-RGD, n=12) compared with the growth of the tumors with infusion of physiological saline solution (NaCl, n=15) or tTF (n=14). The vertical arrows indicate the times of the injections with the respective substances.

c: Inhibition of a human lung carcinoma (CCL185) growing as a xenograft in athymic nude mice by intravenous therapy with tTF fusion proteins (tTF-RGD, n=11) compared with the growth of the tumors in infusion of physiological saline solution (NaCl, n=10) or tTF (n=5). The vertical arrows indicate the times of the injections with the respective substances. Statistical significance was investigated in each case with the Mann-Whitney test for independent groups, P values under 0.05 being regarded as significant. * shows the statistical significance of the difference between tTF-RGD and buffer.

Fig. 35: Macrograph of a mouse bearing an M21 tumor at the end of treatment (day 7) with tTF-RGD fusion protein (A, C) or NaCl (B, D). The difference in size and the different appearance of the tTF-RGD-treated tumors, which in contrast to the apparently vital control tumor show clear signs of necrosis, are readily discernible.

Fig. 36: H-E staining of tumors and organs of mice treated with tTF-RGD and physiological saline solution

Severe thrombosis and necrosis of tumor cells was observed in animals treated with tTF-RGD (A: 200x, B: 400x). Arrows show examples of thromboses in blood vessels of the tumor. No obvious thrombosis or necrosis occurred in animals treated with saline (C: 200x, D: 400x). Arrows show intact blood vessels of the tumor with some erythrocytes. Heart (E), lung (F), liver (G) and kidney of the animals treated with tTF-RGD did not show any visible thrombosis or necrosis.

Fig. 37: Action of tTF-NGR in a fibrosarcoma model

Mice bearing a fibrosarcoma (HT1080) were investigated by magnetic resonance imaging (MRI) without (pre tTF-NGR) and 6 hours after (post tTF-NGR) i.v. administration of tTF-NGR. The high or low vascular volume fraction is shown.

Detailed description of the invention

The problems observed in the prior art were now overcome by fusion polypeptides, which comprise the following peptides:

a) a peptide of 3 to 30 amino acids capable of selectively binding the fusion polypeptide to tumor vessel endothelial cells; and

b) a tissue factor (TF) or a fragment thereof, the tissue factor and the fragment being characterized in that they are able to activate blood clotting when the fusion polypeptide binds to tumor vessel endothelial cells,

wherein the peptides a) and b) are coupled to one another either directly or via a linker having up to 15 amino acids, characterized in that the peptide capable of selectively binding the fusion polypeptide to tumor vessel endothelial cells is coupled to the C-terminus of the peptide capable of activating blood clotting upon binding of the fusion polypeptide to tumor vessel endothelial cells. The present invention further relates to drugs containing corresponding fusion polypeptides and the use thereof for the treatment of tumors.

In addition to sequences a) and b), the fusion polypeptides according to the invention may comprise additional sequences, provided these do not have an adverse effect on the steric conformation of the fusion polypeptide and do not hamper the formation of the enzyme-

substrate complex that triggers blood clotting. The fusion polypeptides according to the invention may for example contain sequences of a His-Tag, which simplify the recombinant expression and purification of the peptide (cf. Examples). The presence of these sequences is not necessary, however. According to a preferred embodiment of the invention, the fusion polypeptide therefore comprises:

a) a peptide of 3 to 30 amino acids capable of selectively binding the fusion polypeptide to tumor vessel endothelial cells; and

b) a tissue factor (TF) or a fragment thereof, the tissue factor and the fragment being characterized in that they are able to activate blood clotting when the fusion polypeptide binds to tumor vessel endothelial cells,

wherein the peptides a) and b) are coupled to one another either directly or via a linker having up to 15 amino acids. According to a particularly preferred embodiment of the invention, the fusion polypeptide comprises:

a) a peptide of 3 to 30 amino acids capable of selectively binding the fusion polypeptide to tumor vessel endothelial cells; and

b) a tissue factor (TF) or a fragment thereof, the tissue factor and the fragment being characterized in that they are able to activate blood clotting when the fusion polypeptide binds to tumor vessel endothelial cells,

wherein the peptides a) and b) are coupled to one another.

According to the invention it was surprisingly shown, that fusion polypeptides from a particularly small peptide capable of selectively binding the fusion polypeptide to tumor vessel endothelial cells, and a peptide capable of activating blood clotting when the fusion polypeptide binds to tumor vessel endothelial cells, are especially advantageous for antivascular tumor therapy. The small size of the polypeptide which permits binding to tumor vessel endothelial cells improves the orientation of the fusion protein to the phospholipid membrane of the endothelial cell. Formation of the enzyme/substrate complex that is essential for blood clotting is not sterically hindered and the tissue factor TF, which can activate blood clotting, is not subjected to a change of conformation.

According to a preferred embodiment of the present invention, the peptide capable of activating blood clotting when the fusion polypeptide binds to tumor vessel endothelial cells is the tissue factor TF with the amino acid sequence shown in SEQ ID NO:1 (Fig. 12). The invention further comprises tissue factor sequences having an amino acid homology of at least 70% or at least 80% to SEQ ID NO:1 (Fig. 12), sequences with a homology of at least 95% being especially preferred. The degree of homology is determined by writing the two sequences one above the other, four gaps on a length of 100 amino acids being possible, to achieve maximum possible agreement of the sequences being compared (cf. Dayhoff, Atlas of Protein Sequence and Structure, 5, 124, 1972). Then the percentage of the amino acid residues of the shorter of the two amino acid chains is determined, which stands opposite to identical amino acid residues on the other chain.

The peptide capable of activating blood clotting in tumor vessels when the fusion polypeptide binds to tumor vessel endothelial cells can moreover be a fragment of the tissue factor TF or a fragment of a sequence homologous to TF. Preferably the fragment has the sequence shown in SEQ ID NO:2 (Fig. 13). The sequence (tTF_{1-218} or tTF for short) shown in SEQ ID NO:2 (Fig. 13) comprises the N-terminal 218 amino acids of TF. Moreover, according to the invention it is also possible to use fragments of tTF that lack several amino acids at the N-terminus or C-terminus, relative to tTF. For example, it is possible to use fragments that lack up to 10 amino acids at the N-terminus (tTF_{11-218}). Furthermore, fragments can be used that lack up to 8 amino acids at the C-terminus (tTF_{1-210}), such as (tTF_{1-214}).

The present invention relates to fusion polypeptides wherein the peptide capable of selectively binding to the endothelial cells of tumor vessels is coupled to the C-terminus of the peptide capable of activating blood clotting. According to the invention, the term "tumor vessel endothelial cells" and the term "endothelial cells in tumor vessels" are used to refer to cells covering the blood vessels of a tumor. It was established according to the invention that the above arrangement ensures orientation of the fusion protein perpendicularly to the phospholipid membrane of the endothelial cell, which is especially advantageous for triggering blood clotting. This orientation corresponds to the natural orientation of the TF during induction of blood clotting. As shown in Fig. 3, very similar Michaelis-Menten kinetics are found with respect to activation of factor X by FVIIa/tTF_{1-218} or FVIIa/tTF_{1-218} fusion proteins for all constructs produced in this way. In the prior art, in contrast, the peptide which activates clotting was coupled to the C-terminus of the targeting molecule (cf. (16)). The fusion polypeptides according to the invention thus differ fundamentally from the peptides used in the prior art.

The peptide capable of selectively binding the fusion polypeptide to tumor vessel endothelial cells can be any peptide which has a length of 3-30 amino acids and binds tumor vessel endothelial cells with high specificity. Corresponding peptides can be isolated from peptide libraries by methods that are usual in the state of the art. They can have a linear or cyclic structure, depending on the peptide library that is chosen.

According to one embodiment of the present invention, the peptides that permit selective binding of the fusion polypeptide to tumor vessel endothelial cells comprise the amino acid sequence RGD or NGR. Both sequences were known in the prior art for their specific binding to integrins, especially $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (RGD peptides), and as cell adhesion motifs (NGR peptides) (cf. (8)). According to the invention it was shown, surprisingly, that these peptides are especially suitable to be part of a fusion polypeptide, the other part of which is a peptide capable of activating blood clotting in tumors when the fusion polypeptide binds to tumor vessel endothelial cells.

Especially advantageous effects were obtained with the linear peptides with the sequences GRGDSP (SEQ ID NO:33), GNGRAHA (SEQ ID NO:34) and GALNGRSHAG (SEQ ID NO:35) and the cyclic peptides with the sequences GCNGRCG (SEQ ID NO:36), GCNGRCVSGCAGRC (SEQ ID NO:37) and GCVLNGRMEC (SEQ ID NO:38). It was demonstrated that fusion polypeptides comprising these sequences and the sequence of the first 218 amino acids of human TF are highly suitable for antivascular tumor therapy. In particular, it was shown that these fusion polypeptides cause significant inhibition of tumor growth or reduce the size of tumors (see Figs. 7 and 8). The observed induction of partial remission of the tumors (cf. Fig. 8) points to anticipation of positive results in human tumor therapy based on the high predictive power of the mouse model (42, 43, 44).

The invention further comprises fusion proteins with cyclic RGD peptides, since cyclization improves the affinity for integrins (as described for example in reference 21).

The present invention further relates to fusion polypeptides having one of the sequences shown in SEQ ID NO:3-8 (Fig. 14-19).

According to another embodiment, the present invention relates to nucleic acids encoding a fusion polypeptide, as described above. Corresponding nucleic acids can, for example, have one of the sequences shown in SEQ ID NO:10-15 (Fig. 21-26).

In yet another aspect, the present invention relates to vectors comprising one of the aforementioned nucleic acids. Corresponding vectors usually also comprise regulatory sequences for expression of the nucleic acid. Said vectors are comprehensively described in the prior art and are available commercially from a large number of companies.

In a further embodiment, the present invention relates to cells comprising one of the aforesaid nucleic acids or vectors. The cells are generally used for expression of the nucleic acid and recombinant production of the fusion polypeptides according to the invention. A large number of cells may find application for this purpose, including *E. coli*, yeast cells and animal cell lines, such as CHO- or COS-cells. Appropriate cells and the use thereof are described comprehensively in the prior art.

The polypeptides of the invention according to claim 1 can further be produced by other suitable methods, for example by chemical coupling of individual peptides. Thus, individual peptides can be produced by methods that are conventional in the state of the art, e.g. by chemical synthesis or by heterologous expression, and are then joined together by coupling.

Finally, the present invention also relates to pharmaceutical compositions comprising the fusion polypeptides, nucleic acids, vectors or cells described above. The pharmaceutical compositions may further comprise pharmaceutically compatible carriers, excipients or adjuvants. Moreover, the polypeptides in said pharmaceutical composition may be present in a modified state, e.g. pegylated, i.e. coupled to a polyethylene glycol molecule.

The fusion polypeptides according to the invention or pharmaceutical compositions containing these fusion polypeptides may be used for the treatment of neoplastic diseases, and especially for antivascular tumor therapy. Neoplastic diseases that may be considered for treatment with the aid of the fusion polypeptides according to the invention or pharmaceutical compositions containing these fusion polypeptides include for example bronchial carcinomas and other tumors of the thorax and mediastinum, breast cancers and other gynecological tumors, colorectal carcinomas, pancreatic carcinomas and other tumors of the gastrointestinal tract, malignant melanomas and other skin tumors, tumors in the head and neck region, prostate carcinomas and other urogenital tumors, sarcomas, endocrine-active tumors, leukemias and Myelodysplastic Syndromes and Hodgkin lymphomas and non-Hodgkin lymphomas.

Further, benign tumors, for example hemangiomas, and neovascularization in diabetic

retinopathy, can also be treated.

Apart from intravenous administration, subcutaneous and intraperitoneal administration of the fusion polypeptides or pharmaceutical compositions is also possible. By packaging in pharmaceutical vehicles, which prevent cleavage of the fusion polypeptides in the gastrointestinal tract, the fusion polypeptides or pharmaceutical compositions may also be administered orally.

It may further be advantageous to combine administration of the fusion polypeptides according to the invention with other therapeutic approaches, e.g. cytotoxic chemotherapy or irradiation. Combination with other active substances, e.g. combination with factor VIIa or doxycycline, is also possible, but preferably combination of the polypeptide according to the invention with factor VIIa or doxycycline is not necessary.

The invention is described in more detail on the basis of the following examples:

Examples

Example 1: Expression and purification of tTF and tTF fusion proteins

The cDNA coding for the N-terminal 218 amino acids of tissue factor TF (designated as tTF hereinafter) was synthesized by the polymerase chain reaction (PCR) using the primers shown in SEQ ID NO:16 and SEQ ID NO:17 (Fig. 27) and cloned into the expression vector pET-30a(+) (Novagen). The recombinant plasmids were transformed in *E. coli* (BL21), expressed and purified (Qiagen Plasmid Kit).

Along with the truncated tissue factor tTF, tTF peptide fusion proteins were constructed, wherein the targeting peptides are first bound to the carboxyl terminal end of the soluble tissue factor tTF. The following linear fusion proteins were constructed:

tTF-GRGDSP (SEQ ID NO:3; Fig. 14; designated tTF-RGD hereinafter; the PCR primers SEQ ID NO:18 and SEQ ID NO:19 (Fig. 28) were used);

tTF-GNGRAHA (SEQ ID NO:4; Fig. 15; designated tTF-NGR hereinafter; the PCR primers SEQ ID NO:20 and SEQ ID NO:21 (Fig. 29) were used);

tTF-GALNGRSHAG (SEQ ID NO:5; Fig. 16; the PCR primers SEQ ID NO:28 and SEQ ID NO:29 (Fig. 33) were used);

In addition, the following cyclic fusion proteins were synthesized:

tTF-GCNGRCG (SEQ ID NO:6; Fig. 17; designated tTF-cycloNGR1 hereinafter; the PCR primers SEQ ID NO:22 and SEQ ID NO:23 (Fig. 30) were used);

tTF-GCNGRCVSGCAGRC (SEQ ID NO:7; Fig. 18; designated tTF-cycloNGR2 hereinafter; the PCR primers SEQ ID NO:24 and SEQ ID NO:25 (Fig. 31) were used);

tTF-GCVLNGRMEC (SEQ ID NO:8; Fig. 19; designated tTF-cycloNGR3 hereinafter; the PCR primers SEQ ID NO:26 and SEQ ID NO:27 (Fig. 32) were used)

All constructs (including tTF) were expressed in the pET30a(+) vector, which mediates the additional expression of an N-terminal affinity tag of 6 histidine residues and a few vector-coded amino acids. With the aid of this affinity tag, the constructs could be purified by affinity chromatography on a nickel-nitrilotriacetic acid column (Ni-NTA, Novagen). The affinity tag is shown in SEQ ID NO:30. SEQ ID NO:31 and SEQ ID NO:32 show, as examples, the complete amino acid sequences of tTF-GRGDSP with affinity tag (SEQ ID NO:31) and tTF-GNGRAHA with affinity tag (SEQ ID NO:32).

The constructs were selected so that, on the basis of the known X-ray crystal structure of the tTF:FVIIa complex (19), vertical orientation of the tTF fusion protein to the phospholipid membrane of the endothelial cells is ensured, which corresponds to the orientation of the native TF. It was further taken into account that the structure selected should not result in the tTF causing any steric hindrance to interaction with FVIIa and the macromolecular substrate FX. Owing to the specificity of the RGD sequence for the $\alpha_v\beta_3$ integrin and of the NGR sequence for CD13 (aminopeptidase N), tumor selectivity is achieved, as these receptors are expressed selectively and specifically at high density on tumor endothelial cells but, apart from a few exceptions, not on resting endothelial cells in normal tissue (see Fig. 1).

tTF and the fusion proteins described tTF-RGD, tTF-NGR, tTF-GALNGRSHAG (SEQ ID NO:5) and tTF-cycloNGR1-3 were transformed and expressed in *E. coli* (BL21) by means of pET30a(+). Transformed, IPTG-induced *E. coli* BL21 DE3 were centrifuged and absorbed in 5-

7 ml lysis buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM $MgCl_2$; 10 µg/ml aprotinin; 2 mg/ml lysozyme)/g pellet and 20 µl Benzonase (Novagen) added. After 90 min incubation at room temperature (RT) and centrifugation at 12 000 g, 20 min, 4°C, the pellet was resuspended and homogenized by ultrasonic treatment in washing buffer (10 mM Tris/HCl, pH 7.5; 1 mM EDTA; 3% Triton X-100). Inclusion bodies were dissolved over night at RT in 2-4 ml/g pellet on denaturing buffer (6 M guanidinium chloride, 0.5 M NaCl, 20 mM NaH_2PO_4, 1 mM DTT). The supernatant from centrifugation (5000 g, 30 min, 4°C) was filtered with a 0.22 µg filter. The constructs were purified until homogeneous on a nickel-nitrilotriacetic acid column (Ni-NTA, Novagen) via the additionally introduced His-Tag sequences of the construct. Purification and folding of the proteins were carried out with the His Bind Buffer Kit (Novagen). This was followed by dialysis against TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.4).

The identity of the proteins was confirmed by SDS-PAGE, Western Blot and mass spectroscopy (see Fig. 2).

Example 2: Functional characterization of tTF and tTF fusion proteins

The functional activity of these fusion proteins with respect to cofactor activity in the activation of factor X to factor Xa via factor VIIa was demonstrated in vitro by Michaelis-Menten analyses. The ability of tTF and of the tTF fusion polypeptides to intensify the specific proteolytic activation of FX via FVIIa in the presence of phospholipids was determined in a slightly modified version of the method described by Ruf (45). For this, 20 µl of each of the following reagents was pipetted in microtiter plates: (a) 50 nM recombinant FVIIa (Novo-Nordisk) in TBS-BSA; (b) 0.16 nM – 1.6 µM tTF/tTF fusion polypeptide in TBS-BSA; (c) 25 mM $CaCl_2$ and 500 µM phospholipid vesicle (phosphatidylcholine / phosphatidylserine, 70/30, M/M; Sigma). After 10 min incubation at room temperature, 20 µl of the natural substrate FX (Enzyme Research Laboratories) was added at a concentration of 5 µM. Then a sample was taken by pipette at one-minute intervals and the reaction was stopped by adding 100 mM EDTA solution. The amount of FXa that formed was measured by addition of the chromogenic substrate Spectrozyme FXa in a Microplate Reader by determining the change in absorption at 405 nm and the parameters for the Michaelis-Menten kinetics were analysed by the method described by Ruf. The results show that both tTF and the tTF fusion polypeptides are functionally active under these conditions (Fig. 3). The Michaelis constants (Km) found for the fusion polypeptides were in the range 0.12-1.2 nM (Fig. 3), and thus in the lower range that is

published for tTF. It can therefore be assumed that the functional activity is unaffected by the fusing of tTF with the peptides.

Example 3: Binding of the tTF fusion proteins to $\alpha_v\beta_3$ *in vitro* and *in vivo*

Binding of tTF-RGD and tTF-NGR to the $\alpha_v\beta_3$ integrin was demonstrated in an ELISA (Enzyme Linked Immunosorbent Assay), by immobilizing purified $\alpha_v\beta_3$ on microtiter plates (see Fig. 4). The specificity of the binding of tTF-RGD to $\alpha_v\beta_3$ was emphasized by the fact that the synthetic peptide with the sequence GRGDSP (SEQ ID NO:33) (from the company Gibco) competitively inhibits the binding of tTF-RGD to $\alpha_v\beta_3$ in this test system (see Fig. 5).

Next the specific binding of tTF-RGD to $\alpha_v\beta_3$ on endothelial cells was evaluated. For this, the differential binding of biotinylated tTF and tTF-RGD to endothelial cells in suspension was analysed by FACS (Fluorescence Activated Cell Sorting). The fact that all endothelial cells held in tissue culture are activated, i.e. express $\alpha_v\beta_3$ molecules, is utilized experimentally. This can be detected by various immunohistochemical methods. A cultivated endothelial cell thus corresponds, in relation to its expression pattern with respect to $\alpha_v\beta_3$, to a tumor endothelial cell. Accordingly, a cultivated endothelial cell can be used as a model system for the specific binding of substances to tumor endothelial cells and also permits predictions to be made concerning the expected toxicity.

Streptavidin-phycoerythrin was used as the detection method. The measured fluorescence intensity for tTF-RGD was higher by a factor of 8 than for tTF (Fig. 6A). Furthermore, the binding of 0.1 μM tTF-RGD to endothelial cells was lowered competitively by 75% by the administration of 1 μM of the synthetic peptide GRGDSP (SEQ ID NO:33) (Fig. 6B). This emphasizes the specificity of the binding of tTF-RGD to RGD-binding receptors on the endothelial cell surface like $\alpha_v\beta_3$.

Example 4: Antitumor effects of the tTF fusion proteins in an animal model

The tTF-RGD and tTF-NGR fusion proteins were evaluated with respect to their effects and side-effects on xenografts of human tumors in athymic nude mice. The models established in our laboratory were used for this (33, 34). The cell lines CCL185 (human adenocarcinoma of the lung) and M-21 (human melanoma) were injected subcutaneously into the flank of male BALB/c nude mice (9-12 weeks old). On attaining a tumor volume of about 50-100 mm^3

(CCL185) or 400-600 mm^3 (M-21), the mice were assigned to four groups at random. Group 1 received only physiological saline solution (NaCl), group 2 tTF, group 3 tTF-RGD, and group 4 tTF-NGR (in each case 1.5-2.0 mg/kg body weight (BW) of the protein). The injections were made in the caudal vein of the animals at intervals of 1-3 days (depending on the growth rate of the particular cell line). Considerable therapeutic activity of the fusion proteins was observed. The tumors of the mice treated with tTF-RGD or tTF-NGR fusion proteins were significantly inhibited in their growth or were reduced in size as far as partial remission in comparison with tTF or NaCl (see Figs. 7 and 8).

To verify the mechanism of action of thrombosis induction in tumor vessels, the following experiment was carried out: the human melanoma cell line was injected into the flank of two male BALB/c nude mice. On attaining a tumor size of approx. 500 mm^3, 2.0 mg/kg BW tTF-NGR or NaCl was injected into the caudal vein. Fig. 9A shows an in-vivo macrograph of the tumor-bearing mouse 20 min after injection of the tTF-NGR fusion protein (left half of the picture) or NaCl (right half of the picture). The macroscopic picture with bluish-livid coloration of the tumor after injection of tTF-NGR indicates tumor necrosis. After 60 min the mice were exsanguinated, the tumor was excised in toto and investigated histologically. Fig. 9B shows the hemorrhagic imbibition of the tumor treated with tTF-NGR as a sign of secondary hemorrhage as a result of incipient tumor necrosis. In contrast, the tumor treated with NaCl appears to be vital (Fig. 9C).

Histological analysis of the melanoma tumor shows microscopically visible thrombus formation in the blood vessels (Fig. 10A-D). This finding verifies the suggested mechanism of anti-tumor effects of tTF-NGR, i.e. induction of thrombi in the blood vessels. The high selectivity of tTF-NGR for tumor blood vessels is demonstrated by the absence of histological detection of clotting and necrosis in normal tissue such as heart, kidney, liver and lung (Fig. 11A-D). Even repeated high doses of tTF-NGR (4 mg/kg BW) did not lead to any visible clot formation or organ toxicity.

<u>Example 5: Antitumor effects of the tTF fusion proteins in the HT1080 tumor animal model</u>

The antitumor activity of the tTF-RGD fusion protein was also investigated in BALB/c nude mice with fibrosarcomas (HT1080). These tumors grow rapidly and are well-vascularized. The results of two experiments are presented in Table 2 and Fig. 34. After the second injection of tTF-RGD, significant inhibition of growth of the HT1080 tumors was observed compared

with control groups. This effect lasted until the end of the experiment on day 7 (P=0.021 for tTF-RGD relative to the buffer control (physiological saline solution), P=0.005 for tTF-RGD relative to tTF). As in the earlier experiments, partial regression of tumor volume was observed in this model.

Table 1: Effect of tTF-RGD on the growth of M21 tumors in mice

Treatment	Mean tumor volume (mm^3)		P relative to buffer	P relative to tTF	n
	Day 0	Day 7			
Buffer	590±77	994±140		ns	9
tTF	558±47	931±147	ns		11
tTF-RGD	585±85	514±81	<0.01	<0.05	7

ns: not significant

Table 2: Effect of tTF-RGD on the growth of HT1080 tumors in mice

Treatment	Mean tumor volume (mm^3)		P relative to buffer	P relative to tTF	n
	Day 0	Day 7			
Buffer	1671± 296	2431±559		ns	15
tTF	1751±269	2335±398	ns		14
tTF-RGD	1725±197	1241±122	<0.05	<0.01	12

ns: not significant

Table 3: Effect of tTF-RGD on the growth of CCL185 tumors in mice

Treatment	Mean tumor volume (mm^3)		P relative to buffer	P relative to tTF	n
	Day 0	Day 7			
Buffer	39±3	467±137		ns	9
tTF	44±8	764±148	ns		5
tTF-RGD	45±5	130±19	<0.01	<0.01	10

ns: not significant

Other tTF fusion proteins can be constructed without any problem by a person skilled in the art on the basis of the disclosure of the present invention. Potential candidates are the peptides TAASGVRSMH (SEQ ID NO:39) and LTLRWVGLMS (SEQ ID NO:40), which bind to NG 2, the murine homolog of the human melanoma proteoglycan (12). Expression of NG 2 is restricted to tumor cells and angiogenic vessels of a tumor (35). Another candidate is the synthetic peptide TTHWGFTL (SEQ ID NO:41), which produces selective and potent inhibition of matrix metalloproteinase-2 (MMP-2) (13). As the integrin $\alpha_v\beta_3$ evidently also binds MMP-2 in an RGD-independent manner, this means that the active enzyme is localized on the surface of the angiogenic blood vessels (36). A construct consisting of tTF and this MMP-2 inhibitory peptide might similarly mediate the selective binding of $tTF_{1\text{-}218}$ to the endothelial cell membrane of tumor vessels.

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